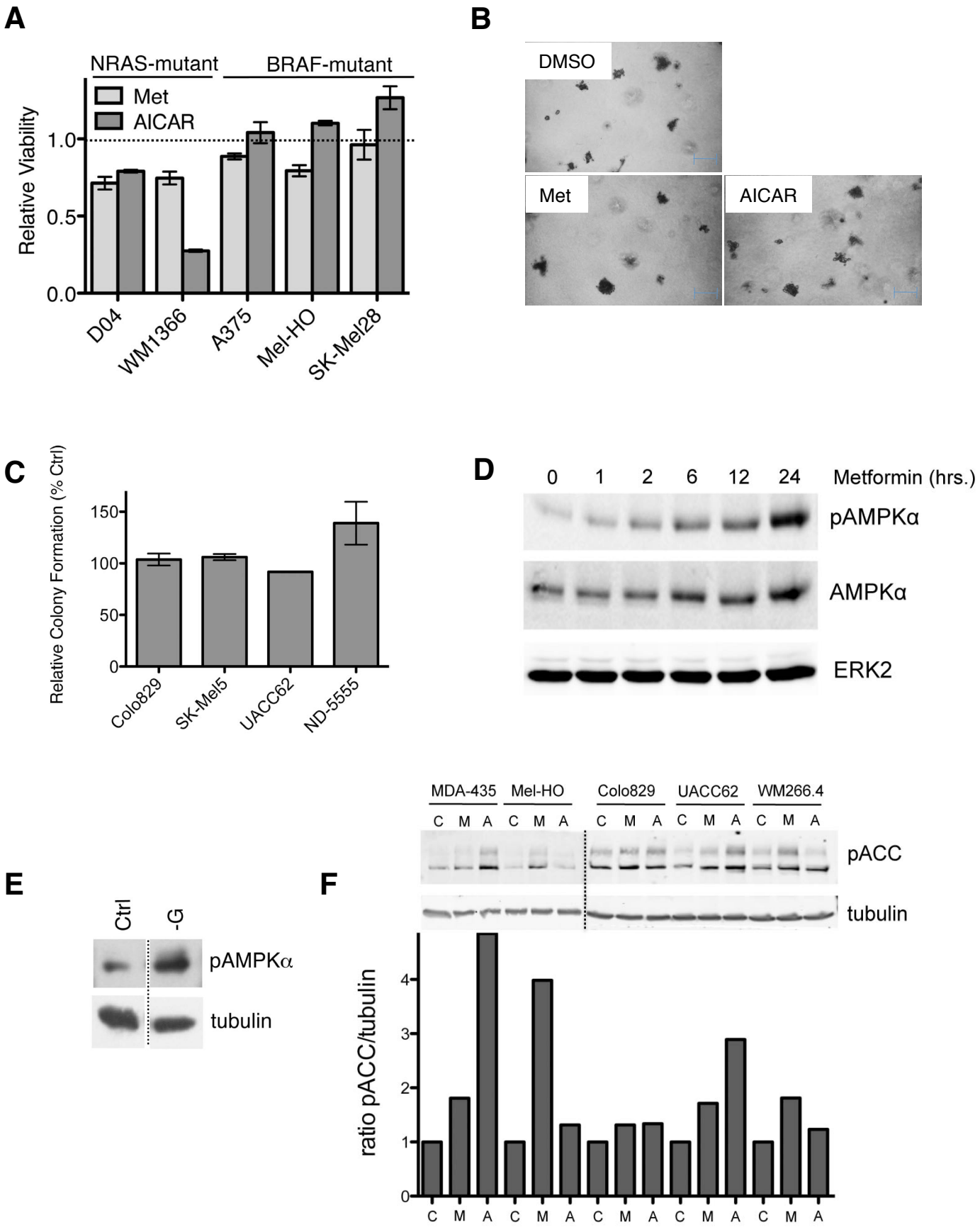
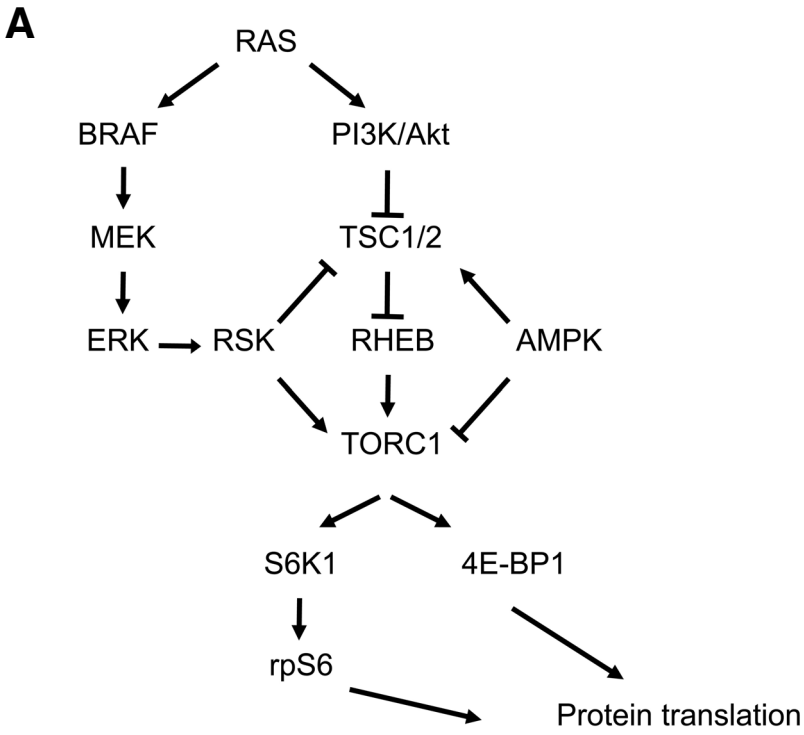
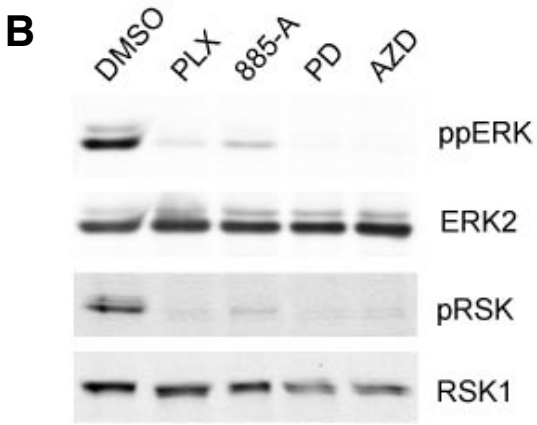
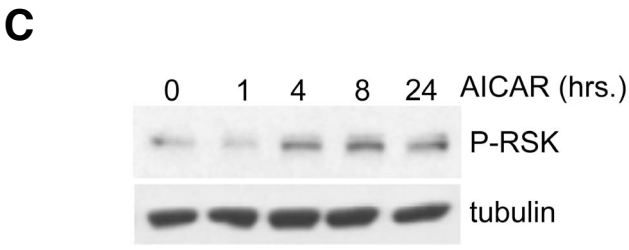
Supplemental Figure 1



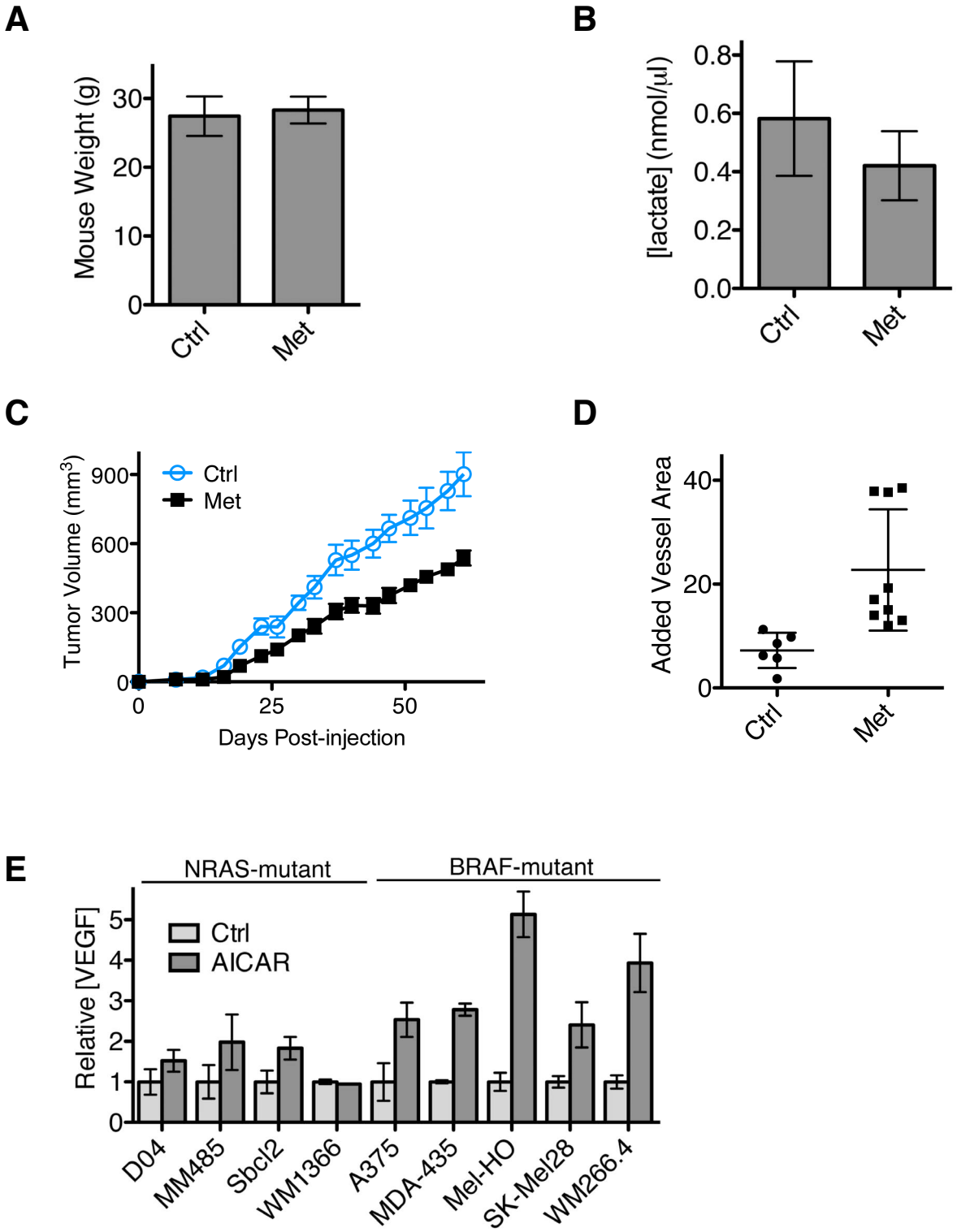
Supplemental Figure 2



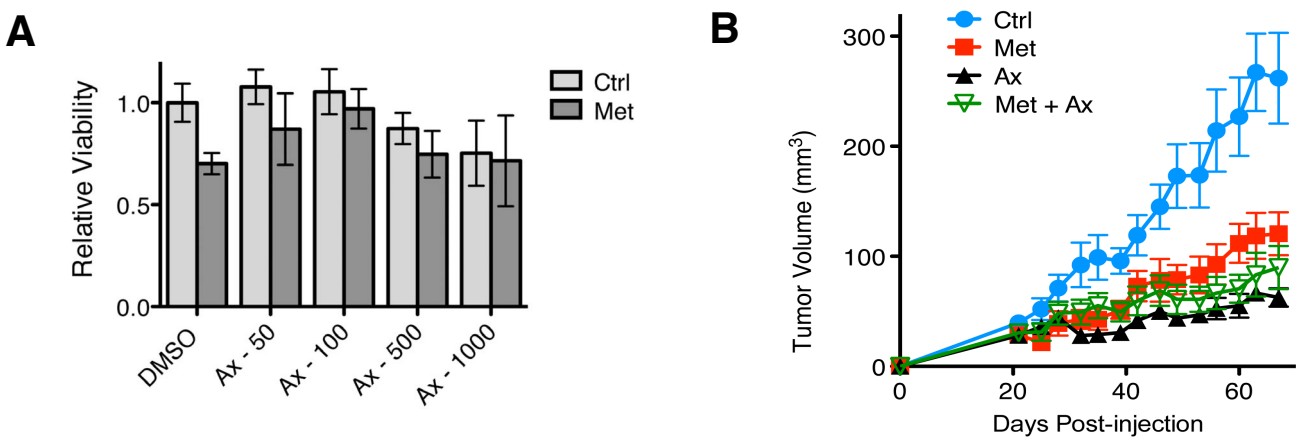




Supplemental Figure 3



Supplemental Figure 4



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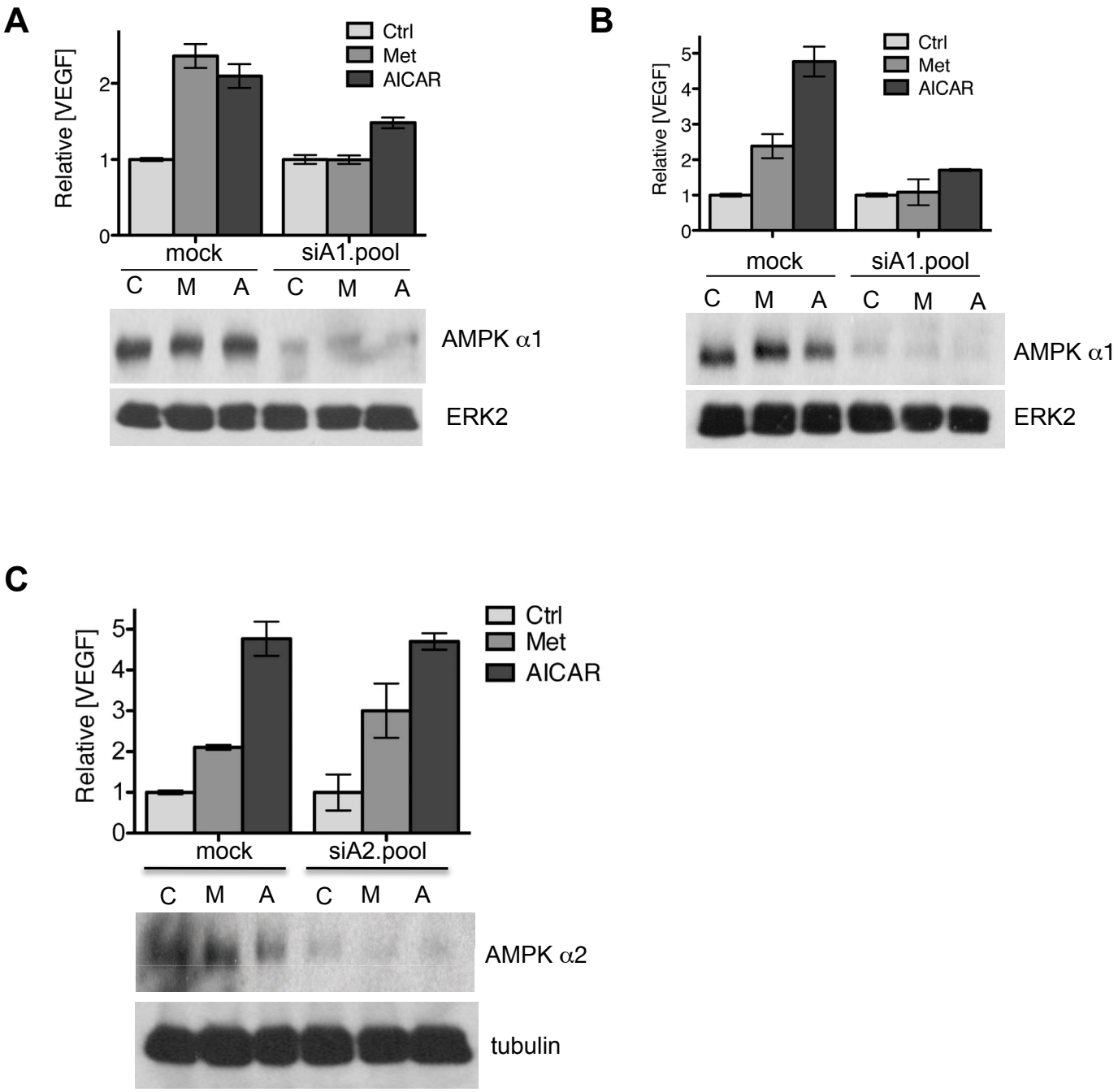
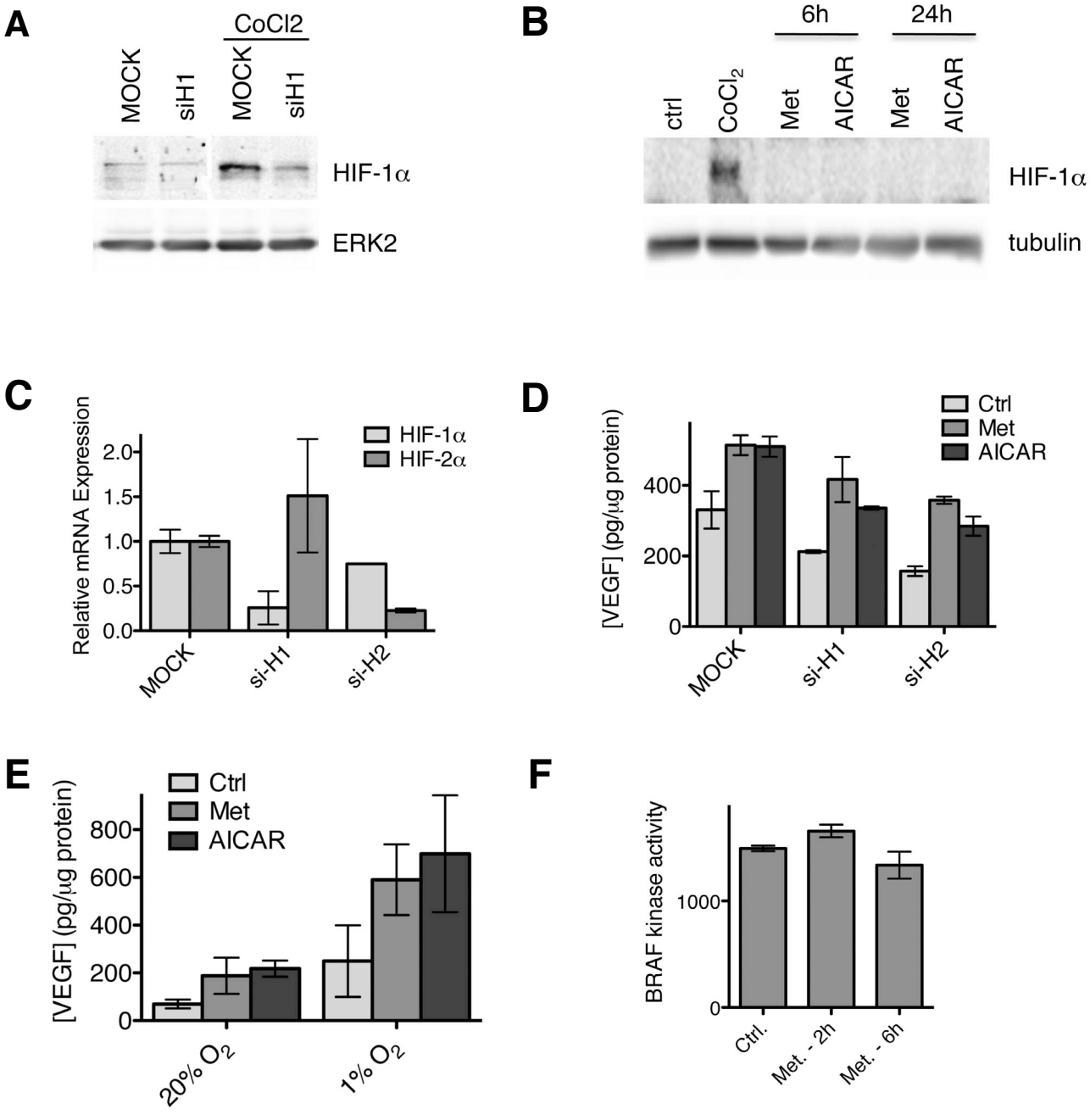
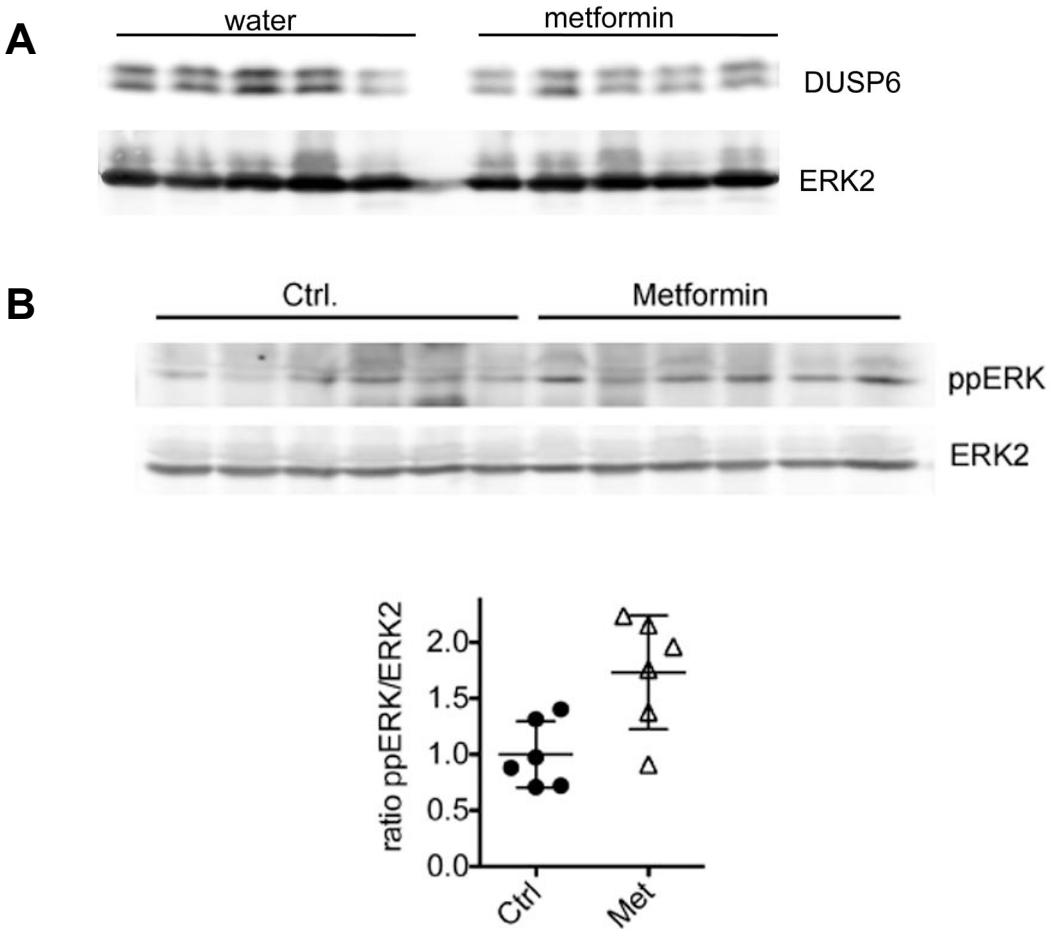


Supplemental Figure 6



Supplemental Figure 7



SUPPLEMENTAL MATERIAL AND DATA

Cell Line	Tumour Type	Source*	Growth Medium	BRAF	NRAS	LKB1
A375	Melanoma	1	DMEM	V600E	WT	WT
Colo829	Melanoma	1	RPMI	V600E	WT	WT
MDA-MB-435	Melanoma/ breast	2	RPMI	V600E	WT	WT
Mel-HO	Melanoma	2	RPMI	V600E	WT	WT
SK-Mel5	Melanoma	3	DMEM	V600E	WT	deleted
SK-Mel28	Melanoma	3	DMEM	V600E	WT	WT
UACC62	Melanoma	2	DMEM	V600E	WT	WT
WM-266.4	Melanoma	1	DMEM	V600D	WT	WT
ND-5555	Melanoma (mouse)	4	DMEM	V600E	WT	WT
D04	Melanoma	5	RPMI	WT	Q61L	WT
MM415	Melanoma	5	RPMI	WT	Q61L	WT
MM485	Melanoma	5	RPMI	WT	Q61R	WT
Sbcl2	Melanoma	6	RPMI	WT	Q61K	WT
WM1366	Melanoma	6	DMEM	WT	Q61L	WT
WM852	Melanoma	6	DMEM	WT	Q61R	WT

Supplemental Table 1. Cell lines used in this study

* See (1-5). The source of cell lines is as follows: **1.** American Type Culture Collection (Manassas, USA) **2.** Institute of Cancer Research tissue bank (NCI-60 collection) **3.** Cell Lines Service (Eppelheim, Germany) **4.** Derived from a tumour from a melanocyte-specific BRAF(V600E) transgenic mouse (5) in our laboratory **5.** Professor Nick Hayward (QIMR, Queensland Australia). **6.** Professor Meenhard Herlyn (The Wistar Institute – Philadelphia, USA). The identity of all cell lines was confirmed by STR profiling (Promega) on an ad hoc basis prior to performing experiments, and repeated for all cell lines after a majority of the experiments were performed (March 2010). All cells were obtained between 2001 and 2010.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1

A. The growth of D04, WM-1366 A375, Mel-HO and SK-Mel28 cells on standard tissue culture plastic in the presence of metformin (2 mM) or AICAR (1 mM) is shown. Cell growth determined in triplicate by SRB assay is expressed relative to DMSO treated controls (fold) with error bars to represent SD from the mean.

B. Photomicrograph (40x magnification) showing representative colonies formed by SK-Mel28 cells in soft agar is shown for cells treated with DMSO, metformin (Met; 5 mM), AICAR (0.5 mM). Bar = 200 μm.

C. Colony formation for Colo829, SK-Mel5, UACC-62 and ND-5555 cells in soft-agar in the presence of metformin (2mM) and AICAR (1mM). Colony numbers are represented relative to DMSO treated controls (%). Error bars: SD from the mean (n=3).

D. Western blot for phospho-AMPKα (pAMPKα) and AMPKα in A375 cells treated with DMSO or 2 mM metformin (Met) (Ctrl) for the times indicated.

E. Western blot for phospho-AMPKα (pAMPKα), AMPKα and ERK2 (loading control) in A375 cells cultured in normal media (Ctrl), or cultured in glucose-free (-G) media for 24 hours.

F. Western blot for phospho-ACC (pACC) and tubulin (loading control) in a panel of BRAF-mutant melanoma cells treated for 12 hours with metformin (5 mM) or AICAR (1 mM). Densitometry of the individual pACC bands is represented by the bar graph, normalized to tubulin levels.

Supplemental Figure 2

A. Schematic diagram of the intersection of BRAF and AMPK with the mTOR signaling pathway.

B. Western blot for phospho-ERK1/2 (ppERK), total ERK2, phospho-RSK (pRSK and total RSK1 in A375 cells treated with vehicle control (DMSO), BRAF inhibitors (1 μM PLX4720 (PLX) or 100 nM 885-A), or MEK inhibitors (1 μM PD184352 (PD) or 1 μM AZD6244 (AZD)) for 4 hours.

C. Western blot for phospho-RSK (pRSK) and tubulin (loading control) in A375 cells treated with AICAR (1 mM) for the times indicated in hours.

Supplemental Figure 3

A. Weight in grams of mice given normal water or water containing 300 mg/kg metformin for 67 days (n=6 for each group).

B. Lactate levels measured in blood serum isolated from mice given normal water or water containing 300 mg/kg metformin for 53 days (n=4 for each group).

C. The growth of D04 cells as tumor xenografts in nude mice treated with metformin (Met) or water (Ctrl) is shown. Error bars represent standard error (n=7).

D. Cumulative vessel area for 3 randomly selected high-powered fields from sections of A375 xenografts from mice treated with water (Ctrl.; n=6) or metformin (Met.; n=9) is shown. Bar: mean; error bars: SD.

E. VEGF-A protein levels in conditioned media from D04, MM485, Sbcl2, WM1366, A375, MDA-MB-435, Mel-HO, SK-Mel28 and WM266.4 cells treated with AICAR for 24h. Error bars: SD from the mean.

Supplemental Figure 4

A. The growth of D04 cells on standard tissue culture plastic in the presence of metformin (Met; 2mM) and/or axitinib (doses in nM as indicated) is shown. Cell growth determined by SRB assay (n=5) is expressed relative to DMSO treated controls (fold) with error bars to represent SD from the mean.

B. The growth of D04 cells as tumor xenografts in nude mice treated with water (Ctrl), metformin (Met; 300 mg/kg/day) and/or axitinib (Ax; 10 mg/kg/day) is shown. Error bars represent standard error from the mean (n=7).

Supplemental Figure 5

A. VEGF-A protein levels in conditioned media from Mel-HO cells transfected with pooled siRNAs specific for AMPKα1 and treated with metformin (M; 2mM) or AICAR (A; 1mM). Error bars: SD from the mean. Levels of AMPKα1 and ERK2 (loading control) as determined by Western blot are shown.

B. VEGF-A protein levels in conditioned media from MDA-MB-435 cells transfected with pooled siRNAs specific for AMPKα1 and treated with metformin (M; 2mM) or AICAR (A; 1mM). Error bars: SD from the mean. Levels of AMPKα1 and ERK2 (loading control) as determined by Western blot are shown.

C. VEGF-A protein levels in conditioned media from A375 cells transfected with pooled siRNAs specific for AMPKα2 and treated with metformin (M; 2mM) or AICAR (A; 1mM). Error bars: SD from the mean. Levels of AMPKα2 and tubulin (loading control) as determined by Western blot are shown.

Supplemental Figure 6

A. Western blot for HIF-1α and ERK2 (loading control) in A375 cells mock treated, or treated with HIF1α specific siRNA (HIF1α si) and treated with $CoCl_2$ (0.1 mM).

B. Western for HIF-1α and tubulin (loading control) in A375 cells treated with $CoCl_2$ (0.1 mM) for 6h, metformin (5 mM) for 6 or 24h, or AICAR (1 mM) for 6 or 24h.

C. mRNA levels for HIF-1α and HIF-2α were assessed 72 after transfection of A375 cells with pooled siRNAs targeting HIF-1α (si-H1) or HIF-2α (si-H2). Results are presented relative to mock transfected cells. Error bars: SD from the mean.

D. VEGF-A protein levels in conditioned media from A375 cells transfected with pooled siRNAs targeting HIF-1α (si-H1) or HIF-2α (si-H2), or mock transfected, and treated with normal media (Ctrl), metformin (Met; 2mM) or AICAR (1mM). Error bars: SD from the mean.

E. VEGF-A protein levels in conditioned media from A375 cells grown for 24h in either a 20% or 1% oxygen incubator, and treated with normal media (Ctrl), metformin (Met; 2mM) or AICAR (1mM). Error bars: SD from the mean.

F. Kinase activity (arbitrary units) of BRAF immunoprecipitated from cells treated with 2 mM metformin for 0, 2 and 6h. Error bars: SD from the mean (n=3).

Supplemental Figure 7

A. Western blot for DUSP6 and ERK2 (loading control) in protein extracts of A375 tumour xenografts from control or metformin-treated animals (see Fig 3A).

B. Western blot for phospho-ERK (ppERK) and ERK2 (loading control) in protein extracts of A375 tumour xenografts from control or metformin-treated animals. Levels were measured by densitometry of individual bands of ppERK on Western blot, and normalized to the corresponding ERK2 band.

SUPPLEMENTAL METHODS

Cell culture techniques - The cell lines used in this study are presented in Supplemental Table 1. An expression plasmid for avian myr-RSK1 was a kind gift of John Blenis. Myristoylated avian RSK1 was excised from pRK7-myr-avRSK1 with HindIII and EcoRI and ligated into pcDNA3.1(+) cut with the same enzymes. To generate cells stably expressing activated RSK, D04 cells were transfected with pcDNA3.1 expressing myristoylated avian RSK (myr-RSK) cloned (6) or empty pcDNA3.1 using lipofectamine2000 and then selected in 1 mg/ml G418. Cells were grown under standard conditions of 20% oxygen, with the exception of hypoxia experiments, where cells were grown in a Whitley H35 hypoxystation incubator at 1% oxygen.

Preparation of cell lysates and Western blotting - Culture medium was aspirated from cells and cells were placed on ice and washed once cold PBS. Cells were scraped into 75-150µl RIPA extraction buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1%NP-40, 1% sodium deoxycholate, 10 mM NaF, 1 mM Na3VO4, 1 µg/ml leupeptin, 1 µg/ml aprotinin) per 35 mm dish and incubated on ice for five minutes. The lysates were sonicated for 15s before protein concentrations were determined by the bicinchoninic acid (BCA) protocol from Pierce (Thermosicentific, UK). Western blots were performed by running samples on SDS-PAGE gels (8-12% acrylamide), transferring proteins to polyvinylidene fluoride membranes, incubating with primary antibodies overnight, followed by addition of fluorescent-labeled secondary antibodies (Li-COR Biosciences) and analyzed on an Odyssey Infrared Scanner (Li-COR Biosciences).

qPCR – RNA was isolated with the RNeasy kit (Qiagen) and reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Sigma) according to the manufacturer's instructions. The real-time PCR was carried out in a final volume of 20 μl containing 1 μl of diluted cDNA sample, 10 μl of 2x Precision Mastermix (PrimerDesign) and 1 μl of 20x TaqMan Gene Expression Assay (Applied Biosystems). Reactions were carried out on an Applied Biosystems 7900HT Fast Real Time Machine and analysis of results was performed using Sequence Detection System software version 2.3. Relative expression of mRNA was calculated using the ΔΔCt method using GAPDH as the internal control.

shRNA – shRNA plasmids targeting human VEGF-A was purchased from SA Biosciences. MDA-MB-435 cells were transfected with plasmids encoding either non-specific sequence (NS: 5'-GGAATCTCATTCGATGCATAC-3'), anti-VEGF-A clone 1 (shV.1: 5'-CAGCTACTGCCATCCAATCGA-3') or anti-VEGF-A clone 3 (shV.3: 5'-GGAGTCCAACATCACCATGCA-3'). Each plasmid possesses a puromycin resistance gene, and stable expression of shRNA constructs was ensured by maintaining cells in 1 μg/ml puromycin. Effectiveness of knockdown was assessed by VEGF-A ELISA.

siRNA – 1.5×10^5 A375 cells per 35 mm diameter well were seeded in 2ml growth medium the day before transfection. The cells were either transfected with non-specific siRNA (5′-AAACCGUCGAUUUCACCCGGG-3'), siRNA targeting BRAF (siB.1: 5'- CAUGAAGACCUCACAGUAAUU -3'; siB.2: 5'-UCAGUAAGGUACGGAGUAAUU -3'), AMPKα1 (siA.1: 5'-

CCAUACCCUUGAUGAAUUA-3'; siA.2: 5'-GAGGAUCCAUCAUAUAGUU-3'), DUSP6 (siD6.1: 5'- UGGCUUACCUUAUGCAGAA -3'; siD6.2: 5'-GACUGUGGCUACCUUAUG -3'), as well as pooled siRNAs (Santa Cruz Biotechnology) targeting AMPKα1, AMPKα2, HIF-1α, HIF-2α, RSK1 or RSK2 using lipofectaime2000 as recommended by the manufacturer (Invitrogen). Briefly, 1 μl of 20 μM siRNA and 5 μl of lipofectamine2000 were combined in a total of 500 μl Opti-Mem serum free medium in RNase-free tubes. The mix was incubated at room temperature for 25 min. before adding the complexes dropwise to the cells. In each case the final concentration of siRNA was 10 nM.

ELISA – Levels of VEGF-A protein in cell culture supernates and protein extracts were quantified using a human-specific sandwich ELISA (R&D Systems). For drug treatments, 1.5 ml of fresh media containing the drug was added, and supernates collected after 24h. For siRNA experiments, 1.5 ml of fresh media was added 48h after transfection, and supernates collected after an additional 24h. Concentrations of VEGF-A in the culture medium were determined as compared to a standard curve and normalized to the amount of protein from lysates collected from the corresponding cells. For analysis of VEGF-A in xenograft tumor samples, tumor tissue was homogenized in an equal volume of RIPA buffer using the precellys 24 tissue homogenizer (Bertin Technologies). 10 μg of total protein was diluted in 100 μl of PBS before analysis by ELISA.

Soft agar Soft agar assays were performed as described previously (7). Briefly, cells were seeded at a concentration of 6×10^3 cells/35-mm dish. Bottom layers were made up of 0.4% agar in 10% FBS and DMEM. Cells were resuspended in a top layer of

0.2% agar in 10% FBS and DMEM. Indicated concentrations of drug were included in each layer. Cells were fed every other day by placing 2 drops of medium on the top layer, including drug treatment as required. After 2 weeks at 37°C, the number of single cells and colonies/high-power field were counted. Results were formulated as a percentage of macroscopic (>0.1-mm) colonies formed/total number of cells plated. Cell lines were examined at ten sites per well in a minimum of three separate soft agar experiments. The statistical significance of differences in the respective groups was evaluated using the Student's t test; P values of <0.05 were considered to be of statistical significance.

Cell Viabililty Assays

The sulphrodamine B (SRB) assay was performed as described previously (Whittaker (8). 1500-2000 cells were plated in 96-well tissue culture plates. The following day cells were incubated with the indicated drugs and grown for 96h at which time cells were fixed in 10% (wt/vol) trichloroacetic acid, dried for 1h at 37°C and stained for 10 min in SRB. Excess dye was removed by washing repeatedly with 1% (vol/vol) acetic acid. 10 mM Tris base solution was added for 30 min. to solubilize dye-protein complexes, and optical density was determined at 510 nm using a microplate reader. All results are expressed relative to vehicle-treated control cells.

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